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# *In vitro* antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam.

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ABSTRACT

**Objective:** To elucidate the antioxidant activity and total phenolic content (TPC) of ethyl acetate extracts of endophytic fungi isolated from *Eugenia jambolana* by three different antioxidant assays.

**Methods:** Twenty one different endophytic fungal extracts were screened for presence of various phytochemicals, TPC and *in vitro* antioxidant activity. TPC was tested by Folin-Ciocalteau reagent based assay. DPPH free radical scavenging, hydrogen peroxide scavenging and reducing power assays were used to evaluate the antioxidant activity.

**Results:** Alkaloids, phenols, flavonoids, saponins, and terpenes were the main phytochemicals presents in all 21 endophytes. A significant positive correlation was found between antioxidant activity and TPC in fungal extracts. There is 36% endophytic extracts having high phenolic content exhibited potent antioxidant activity. *Chaetomium* sp., *Aspergillus* sp., *Aspergillus peyronelii* and *Aspergillus niger* strain showed the highest antioxidant activity ranging from 50% to 80% having 58 mg/g to 60 mg/g GAE total phenolics. Ascorbic acid used as a standard showed 90% reducing potential.

**Conclusions:** The results reveal that metabolites produced by endophytic fungi isolated from *Eugenia jambolana* can be a potential source of novel natural antioxidant compounds.

## 1. Introduction

The field of free radical chemistry is gaining more attention these days. Free radicals are reactive oxygen and nitrogen species which are generated by various physiological processes in the body. Uncontrolled generation of free radicals leads to attack on membrane lipids, proteins, enzymes and DNA causing oxidative stress and ultimately cell death. These ROS are responsible for many degenerative human diseases like diabetes mellitus, cancer, neurodegenerative disorders, Alzheimer's disease, Parkinson's disease, atherosclerosis, ageing and inflammatory diseases[1].

In human body there are various enzyme systems for free radical scavenging but micronutrients like vitamin E, beta-carotene and vitamin C are the major antioxidants. These must be provided in diet as body cannot produce these nutrients[2]. Protection against free radicals can be enhanced by taking sufficient amounts of exogenous antioxidants. An antioxidant is a stable molecule which donates an electron to a rampaging free radical and terminates the chain reaction before vital molecules are damaged. Free radical scavenging property of antioxidants delays or inhibits cellular damage[3].

All higher plants are hosts to one or more endophytic microbe on this earth. Endophytic fungi are microbes that reside in living plant tissues without causing any immediate harm to their host[4]. They are highly diverse microorganisms which are chemical synthesizers inside host plants[5]. A lot of work has been done on the bioactive potential of endophytes, such as antiviral, anticancer, antidiabetic and antimicrobial effects, but very little is known about their antioxidant capacity[6].

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*Eugenia jambolana (E. jambolana)* Lam. (Syn. *Syzygium cumini*) commonly known as Jamun or black plum is an integral part of indigenous medicine system of India to treat diabetes. Traditionally all parts of Jamun such as fruits, leaves, seeds and bark are used in Ayurvedic medicine. Jamun plant has been reported for a wide range of medicinal properties such as antioxidant, anti-inflammatory, neuropsycho-pharmacological, anti-microbial, anti-HIV, anti-diarrheal, antifertility, gastroprotective, anti-ulcerogenic and radio-protective activities[7]. No reports are available on endophytic fungi and their antioxidant properties isolated from *E. jambolana*.

In present study, *in vitro* antioxidant potential, total phenolic content and preliminary screening of bioactive constituents of ethyl acetate crude extracts of endophytic fungi isolated from *E. jambolana* were investigated.

## 2. Materials and methods

### 2.1. Isolation of endophytic fungi

Plant samples (leaf, petiole and stem tissue) were washed thoroughly in running tap water and rinsed with double distilled water. All samples were surface sterilized and placed on Petri dishes containing potato dextrose agar supplemented with 150 mg/L streptomycin. Plates were incubated at (26±1) °C until fungal growth was initiated. The growing tips of fungal mycelia were transferred to new potato dextrose agar plates for pure culture. All fungi were morphologically and molecularly identified.

### 2.2. Preparation of extracts

The endophytes were grown in potato dextrose broth media for 10 d at 27 °C on a shaker at 160 r/min. The mycelium were filtered and dried. The dried powdered materials were extracted with organic solvent ethyl acetate (1:10) by using cold percolation for 48-72 h. The obtained extracts were filtered by using Whatman No. 1 filter paper and then concentrated under vacuum at 40 °C by using a lyophilizer.

### 2.3. Phytochemical screening of endophytic fungal extracts

Twenty one crude endophytic fungal extracts were subjected to phytochemical analysis for the presence of secondary metabolites like alkaloids, amino acids, carbohydrates, tannins, phenols, flavonoids, saponins and terpenes according to standard methods[8,9].

### 2.4. Determination of total phenolic content

Total phenol content of ethyl acetate extracts of endophytic fungi was estimated using Folin-Ciocalteau reagent based assay using gallic acid as standard[10]. Each extract was dissolved in methanol (1 mg/mL) and 500 μL of (50%) Folin -Ciocalteau reagent was added followed by the addition of 1.5 mL of 20% of $Na_2CO_3$. Final volume was made 5 mL by adding distilled water. The mixture was incubated at room temperature for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-vis spectrophotometer. The same procedure was repeated to 1 mL aliquots of 20, 40, 60, 80, 100 μg/mL methanolic gallic acid solutions used as standard for calibration curve. Total phenolic value was obtained from the regression equation: $y=0.056x+1.454$ with $R^2=0.9967$ and expressed as mg/g gallic acid equivalent using the formula:

$C=cV/M$

Where C is total content of phenolic compounds in mg/g GAE; c is the concentration of gallic acid established from the calibration curve; V is volume of extract and M is the weight of fungal extract.

### 2.5. Antioxidant assays

Three different assays including free radical scavenging DPPH assay, hydrogen peroxide scavenging assay, and reducing power assay were used to evaluate the antioxidant potential of endophytic fungal extracts. Each experiment was done in triplicate and mean values were taken.

#### 2.5.1. DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity

To check the antioxidant activity through free radical scavenging by the test samples, the change in optical density of DPPH radicals was monitored. Endophytic fungal extracts at 1 mg/mL concentration were used. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. When a substrate that can donate a hydrogen atom added in DPPH solution, it was reduced to a yellow colored product, diphenylpicryl hydrazine. DPPH solution (0.5 mmol/L) was prepared in 95% methanol. The sample extract (0.2 mL) was diluted with methanol. A total of 2 mL of DPPH solution (0.5 mnol/L) was added in the test sample and incubated for 30 min at room temperature in darkness. After 30 min, the absorbance was measured at 517 nm[11]. Free radical scavenging activity was expressed as a

percentage.

The percentage of the DPPH radical scavenging was calculated as:

Inhibition of DPPH radical (%)=[(control absorbance−extract absorbance)/(control absorbance)]×100

### 2.5.2. Hydrogen peroxide scavenging ($H_2O_2$) assay

The ability of fungal extracts to scavenge hydrogen peroxide was estimated by following the method of Ruch *et al*[12]. A solution of hydrogen peroxide (40 mmol/L) was prepared in phosphate buffer (50 mmol/L, pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Endophytic fungal extracts (1 mg/mL) in distilled water was added to hydrogen peroxide and absorbance at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated as follows:

Scavenged $H_2O_2$ (%)=[($A_i$ −$A_t$)/$A_i$]×100

Where $A_i$ is the absorbance of control and $A_t$ is the absorbance of test samples.

### 2.5.3. Reducing power assay

The reductive potential of the extract was determined according to the method of Oyaizu[13]. Different extracts and standard (1 mg/mL) in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v) was added to the mixture, which was then centrifuged for 10 min at 3 000 r/min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophotometer. High absorbance value of the reaction mixture indicates greater reductive potential.

All experiments were performed in triplicates. Results were reported as mean±SD.

## 3. Results

### 3.1. Phytochemical screening

Screening of various phytochemicals of crude extracts reveals a good concentration of alkaloids, phenols, tannins, amino acids, carbohydrates, saponins, terpenes, flavonoids and sterols in endophytic fungi as shown in Table 1. These chemical compounds are responsible for different medicinal properties of extracts.

**Table 1**

Qualitative analyses of the phytochemical components of endophytic fungal extracts.

| Fungal endophyte | Phytochemicals present |
|---|---|
| *Fusarium* sp. | Phenols, amino acids, carbohydrates, saponins, terpenes |
| *Coprinopsis cinerea* | Alkaloids, phenols, tannins, amino acids, saponins, terpenes, flavonoids |
| *Penicillium spinulosum* | Alkaloids, phenols, terpenes, flavonoids |
| *Aspergillus flavus* | Alkaloids, phenols, terpenes, flavonoids, amino acids, carbohydrates |
| Unknown | Alkaloids, phenols, amino acids, carbohydrates, flavonoids |
| *Isaria tenuipes* | Alkaloids, terpenes |
| *Aspergillus* sp. | Phenols, amino acids, saponins, terpenes, flavonoids |
| *Aspergillus peyronelii* | Alkaloids, phenols, amino acids, carbohydrates, terpenes |
| *A. niger* | Alkaloids, phenols, amino acids, saponins, terpenes |
| *Aspergillus tubingensis* | Alkaloids, tannins, carbohydrates, saponins, sterols |
| *Curvularia lunata* | Amino acids, carbohydrates saponins, sterols |
| *Alternaria alternata* | Amino acids, carbohydrates, terpenes |
| *Syncephalastrum racemosum* | Alkaloids, phenols, amino acids, carbohydrates, sterols |
| *Choanephora* sp. | Tannins, amino acids, carbohydrates, terpenes |
| *Chaetomium* sp. | Alkaloids, phenols, amino acids, carbohydrates, flavonoids. |
| *Trichoderma longibrachiatum* | Alkaloids, phenols, amino acids, carbohydrates, terpenes, flavonoids |
| *Aspergillus japonicas* | Tannins, amino acids, carbohydrates, terpenes |
| *Aspergillus terreus* | Alkaloids, phenols, carbohydrates, saponins, terpenes |
| *Paecilomyces formosus* | Alkaloids, phenols, amino acids, carbohydrates |
| *A. niger* strain | Alkaloids, phenol, amino acids, carbohydrates, terpenes |
| *Aspergillus aff. fumigatus* | Amino acids, carbohydrates, terpenes |

### 3.2. Determination of total phenolics

There was a wide range of total phenolic concentrations in endophytic fungal extracts of ethyl acetate as shown in Table 2. The values varied from 4.20 to 60.13 mg GAE/g of dry weight. The highest concentration of phenols was observed in extract of *Chaetomium* sp. (60.13±0.41 mg GAE) followed by *Aspergillus niger* (*A. niger*) strain. Whereas *Fusarium* sp., *Curvularia lunata* and *Syncephalastrum racemosum* extracts contained considerably least concentration of phenols.

**Table 2**

Total phenolic contents of endophytic fungal extracts.

| Fungal endophyte | Total phenolic content (mg of GAE/g of extract) |
|---|---|
| *Fusarium* sp. | 4.46±0.15 |
| *Coprinopsis cinerea* | 26.70±0.26 |
| *Penicillium spinulosum* | 23.90±0.20 |
| *Aspergillus flavus* | 48.93±0.25 |
| Unknown | 33.66±0.35 |
| *Isaria tenuipes* | 4.25±0.20 |
| *Aspergillus* sp. | 55.43±0.15 |
| *Aspergillus peyronelii* | 56.83±0.76 |
| *A. niger* | 23.16±0.25 |
| *Aspergillus tubingensis* | 24.43±0.15 |
| *Curvularia lunata* | 4.20±0.15 |
| *Alternaria alternata* | 23.43±0.25 |
| *Syncephalastrum racemosum* | 4.23±0.15 |
| *Choanephora* sp. | 22.97±0.30 |
| *Chaetomium* sp. | 60.13± 0.41 |
| *Trichoderma longibrachiatum* | 23.43±0.20 |
| *Aspergillus japonicas* | 9.66±0.15 |
| *Aspergillus terreus* | 41.20±0.40 |
| *Paecilomyces formosus* | 33.83±0.35 |
| *A. niger* strain | 58.46±0.15 |
| *Aspergillus aff. fumigatus* | 19.83±0.20 |

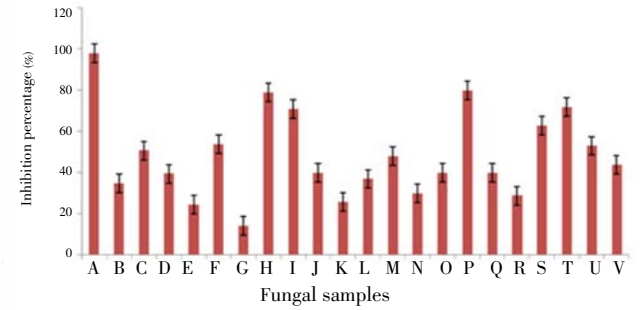


**Figure 1.** DPPH free radical scavenging activity of fungal samples and ascorbic acid.
A: control, B: *Fusarium* sp., C: *Coprionopsis cinerea*, D: *P. spinulosum*, E: *A. flavus*, F: unknow, G: *Isaria tenuipes*, H: *Aspergillus* sp., I: *A. peyron*, J: *A. niger*, K: *A. tubingensis*, L: *Curvulara lunatara*, M: *Alternaria alternata*, N: *S. racemosum*, O: *Choanophora* sp., P: *Choanophora* sp., Q: *T. longibrachiatum*, R: *A. japonicus*, S: *A. terreus*, T: *A. niger* strain, U: *P. formosus*, V: *A. aff. funigatus*.

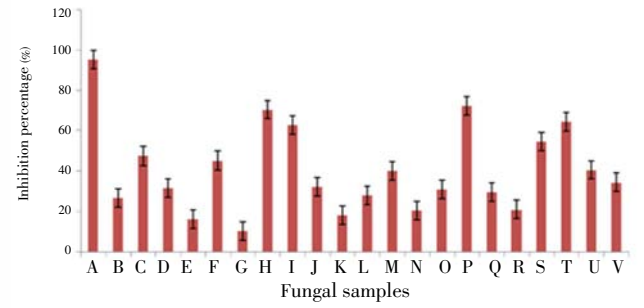


**Figure 2.** Hydrogen peroxide scavenging activity of fungal samples and ascorbic acid.
A: control, B: *Fusarium* sp., C: *Coprionopsis cinerea*, D: *P. spinulosum*, E: *A. flavus*, F: unknow, G: *Isaria tenuipes*, H: *Aspergillus* sp., I: *A. peyron*, J: *A. niger*, K: *A. tubingensis*, L: *Curvulara lunatara*, M: *Alternaria alternata*, N: *S. racemosum*, O: *Choanophora* sp., P: *Choanophora* sp., Q: *T. longibrachiatum*, R: *A. japonicus*, S: *A. terreus*, T: *A. niger* strain, U: *P. formosus*, V: *A. aff. funigatus*.

### *3.3. Antioxidant activity*

In present study, 21 ethyl acetate crude fungal extracts were investigated for antioxidant potential by using three different methods. All 21 extracts showed antioxidant activity up to varying extent. Nine (36%) fungal extracts showed significant antioxidant activity ranging from 50% to 80%. Remaining extracts showed a narrow spectrum of activity ranging from 14% to 40%. Fungal extracts having antioxidant potential also had a good amount of total phenols as tested by Folin-Ciocalteau reagent based assay.

#### *3.3.1. DPPH radical scavenging activity*

The reaction was visible as a color change from purple to yellow. *Chaetomium* sp. and *Aspergillus* sp. showed a high antioxidant capacity value of 80% where as *Isaria tenuipes* showed the least antioxidant activity (14%). *Aspergillus peyronelii* (*A. peyronelii*) and *A. niger* strain were having 71% and 72% of reducing potential respectively. Ascorbic acid was taken as standard showing 98% antioxidant activity. Percentage of DPPH radical scavenging activity of endophytic fungi and ascorbic acid is shown in Figure 1.

#### *3.3.2. Hydrogen peroxide scavenging ($H_2O_2$) assay*

As shown in Figure 2, reduction potential of fungal extracts in $H_2O_2$ assay ranged from 75% to 10%. Reducing activity of ascorbic acid was 90%. *Chaetomium* sp. and *Aspergillus* sp. extracts were showing 75% activity. These were followed by *A. peyronelii* and *A. niger* strain which showed 70% reducing capacity.

#### *3.3.3. Reducing power assay*

In reducing power assay reducing ability was measured by change of $Fe^{3+}$ to $Fe^{2+}$. *Chaetomium* sp. and *Aspergillus* sp. had high absorbance values that indicated their greater reductive potential and electron donor ability for stabilizing free radicals. Activity of all fungal extracts and ascorbic acid with respect to their absorbance values are represented in Figure 3.

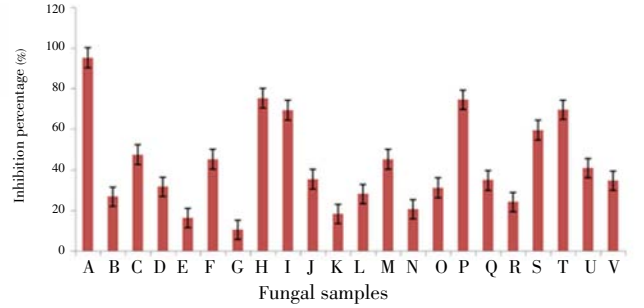


**Figure 3.** Reducing power activity of fungal samples and ascorbic acid.
A: control, B: *Fusarium* sp., C: *Coprionopsis cinerea*, D: *P. spinulosum*, E: *A. flavus*, F: unknow, G: *Isaria tenuipes*, H: *Aspergillus* sp., I: *A. peyron*, J: *A. niger*, K: *A. tubingensis*, L: *Curvulara lunatara*, M: *Alternaria alternata*, N: *S. racemosum*, O: *Choanophora* sp., P: *Choanophora* sp., Q: *T. longibrachiatum*, R: *A. japonicus*, S: *A. terreus*, T: *A. niger* strain, U: *P. formosus*, V: *A. aff. funigatus*.

All three assays revealed that crude extracts of *Chaetomium* sp., *Aspergillus* sp., *A. peyronelii and A. niger* strain possessed maximum antioxidant potential while *Isaria tenuipes* showed least antioxidant activity in all the assays.

## 4. Discussion

Medicinal plants usually harbour endophytes with similar secondary metabolites and medicinal activities[14]. *E. jambolana* is a well known Indian medicinal plant with diverse phytochemicals like flavonoids, anthocyanines, terpenes, aliphatic acids, phenolics and phytosterols which contribute to its medicinal properties[15].

In this study, preliminary phytochemical investigation of ethyl acetate extracts of endophytic fungi associated with *E. jambolana* confirms the presence of alkaloids,

phenols, flavonoids, saponins, and terpenes. Phenols and terpenes are the main chemical constituents responsible for reducing lipid peroxidation and hence act as primary and secondary antioxidants[16,17]. In this study, extracts having high phenolic content also showed good antioxidant activity. Previous studies conclude that there is a linear correlation between total phenolic content and antioxidant potential of any sample[18]. The free radical scavenging ability of phenols is attributed to the occurrence of hydroxyl groups. Phenols and alkaloids were the major phytochemical constituents of endophytes in the study which supports the views of Huang *et al*[19].

Ethyl acetate extraction is most efficient method of isolating fungal secondary metabolites[20]. Ethyl acetate as an extraction solvent selectively extracts low molecular weight phenolic compounds and high molecular weight polyphenols[21].

Among three different assays used, DPPH free radical scavenging assay is a basic and most widely used assay. This method is considered most accurate screening method used to evaluate the antioxidant activity of samples[22]. In reducing power assay, reducing ability of a compound depends on the electron donor and free radical quenching capacity[23]. Reducing agents hinder lipid peroxidation as they donate a hydrogen atom and stop the chain reaction which causes membrane lipid damage[24]. Hydrogen peroxide itself is not very reactive but sometimes it rapidly decomposes into oxygen and water producing hydroxyl radicals ($OH^-$) that causes DNA damage[25]. This results indicated that scavenging activity by $H_2O_2$ was correlated with the scavenging activity by DPPH and reducing power assay.

The results of this study link with some previous finding of endophytic fungi and their antioxidant activity. A list of endophytic fungi isolated from a number of medicinal plants have been claimed to possess antioxidant potential. There is 22% of endophytic fungi extract isolated from five *Garcinia* species plants exhibited antioxidant activities[26]. Endophytes of *Salvadora oleoides*, *Tabebuia argentea* showed antioxidant potential in different assays[27,28]. The endophytic fungi of *Nerium oleander* L. and liverwort *Scapania verrucosa* were shown to have excellent antioxidant capacity[29,30].

Antioxidant activity of *Chaetomium* sp. isolated from wheat (*Triticum durum*) was 38% by using the β-carotene/linoleic acid system oxidation[31]. *Chaetomium* sp. isolated from *Nerium oleander* also possessed the highest antioxidant capacity and phenolic content (13.95±0.11) mg/GAE[29]. But in this study, *Chaetomium* sp. showed 80% antioxidant capacity. This can be attributed to its high phenolic value *i.e.* (60.13±0.41) mg/GAE compared with previous studies.

Ethyl acetate extract of *Aspergillus terreus* isolated from *Ocimum sanctum* exhibited 34.83% antioxidant activity with (14.96±0.07) mg/g GAE phenolic content[32]. In this results, *Aspergillus terreus* showed 63% encouraging antioxidant activity having (41.2±0.40) mg/g GAE.

Fungal endophytes are a store house of novel secondary metabolites including antibiotic, antioxidant, anticancer and immunosuppressant compounds[6]. *Cephalosporium* sp., an endophytic fungus isolated from the root of *Trachelospermum jasminoides* (Apocynaceae) produce a phenolic compound (graphislactone A) with strong free radical scavenging and antioxidant activity[33]. Two antioxidants, pestacin and isopestacin are produced by *Pestalotiopsis microspora*, an endophytic fungus residing in *Terminalia morobensis* in Papua New Guinea[34]. Another antioxidant compound phenylpropanoid amide has been isolated from endophytic fungus *Penicillium brasilianum* that reside in *Melia azedarach*[35]. The results of this study represent that endophytic fungi may serve as a potential source of natural antioxidants. This is the first report on the antioxidant activity of endophytic fungi isolated from *E. jambolana*. This study will provide an introduction to more comprehensive work on bioactive compounds produced by these endophytes.

## Conflict of interest statement

We declare that we have no conflict of interest.

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